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A New Form of *Gastrodia pubilabiata* (Orchidaceae)

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A blackish brown color is a feature of the mycoheterotrophic orchid *Gastrodia pubilabiata*. Here, we report a new form of *G. pubilabiata* with a tinge of red color. A total of ten plants showed a reddish plant body, and this phenotype seems to be stable in two localities. Based on analyses of plant morphology, distribution and pigment, together with color identification using the Munsell color order system, we describe the reddish plants as *G. pubilabiata* f. *castanea*.

Key words: *Drosophila*, *Gastrodia pubilabiata*, mycoheterotrophic orchid, new forma, Orchidaceae, pollinator

Gastrodia R. Br. (Orchidaceae) is a genus of mycoheterotrophic orchids distributed in warm areas of Madagascar, Asia and Oceania (Paul & Molvray 2005, Chung & Hsu 2006). The approximately 50 species are characterized by either fleshy tubers or a coralloid underground stem, the absence of leaves, the union of sepals and petals, and the two mealy pollinia lacking caudicles (Paul & Molvray 2005, Chung & Hsu 2006, Chen *et al*. 2009, Cribb *et al*. 2010, Hsu & Kuo 2010, 2011, Hsu *et al*. 2012, Suetsugu 2013, 2014, Suetsugu *et al*. 2014). The length of the stem and the morphology of the floral organs, such as the lip, column, and calyx tube, are varied in each species of *Gastrodia*. One species, *G. verrucosa* Blume (*G. verrucosa* group), is 3–15 cm tall during flowering, but elongation occurs during the fruiting period and the stem reaches 30–50 cm in height (Schlechter 1911, Tuyama 1967, 1982, Chung & Hsu 2006, Suetsugu 2013, 2014). In contrast, *G. elata* Blume (*G. elata* group) is 60–100 cm tall during flowering.

Thirteen species of *Gastrodia* (*G. elata, G. javanica* (Blume) Lindl., *G. boninensis* Tuyama, *G. confusa* Honda & Tuyama, *G. nipponica* (Honda) Tuyama, *G. pubilabiata* Y. Sawa, *G. shimizuana* Tuyama, *G. gracilis* Blume, *G. clausa* T. C. Hsu, S. W. Chung & C. M. Kuo, *G. takeshimensis* Suetsugu, *G. uraiensis* T. C. Hsu, C. M. Kuo, *G. fontinalis* T. P. Lin and *G. flexistyloides* Suetsugu) have been reported in Japan (Honda & Tuyama 1939, Tuyama 1939, Tuyama 1941, 1952, 1956, 1966, 1967, 1982, Garay & Sweet 1974, Hatusima 1975, Sawa 1980, Kobayashi &Yukawa 2001, Suetsugu *et al*. 2012, 2013, 2014, Suetsugu 2013, 2014, 2015a, 2015b). Most of them flower in the spring or summer, but *G. pubilabiata* and *G. confusa* flower in the autumn. The latter two species can be easily distinguished by stem height, presence or absence of hairs on the lip, the morphology of calli at the base of the lip, and the color of the flowers (Sawa 1980, Kobayashi &Yukawa 2001). *Gastrodia pubilabiata* (Fig. 1A) and *G. confusa* have been reported in Japan and Taiwan (Sawa 1980, Kobayashi & Yukawa 2001, Leou 2000, Chen *et al*. 2009), and in Korea, Japan and Taiwan (Honda & Tuyama 1939, Tuyama 1967, Chen *et al*. 2009).

Mycoheterotrophic orchids have various body colors. Many forms have been proposed based on

FIG. 1. A: *Gastrodia pubilabiata* (Kamo, Tosayamada-cho, Kochi). B–C: *G. pubilabiata* f. *castanea* (Machida, Tosayamada-cho, Kochi). D: Flowering plants of *G. pubilabiata* (left) and *G. pubilabiata* f. *castanea* (right). E–F: Fruiting plants of *G. pubilabiata* f. *castanea* (E) and *G. pubilabiata* (F). G: Comparison of fruiting plants of *G. pubilabiata* (left) and G. *pubilabiata* f. *castanea* (right). Scale bars = 1 cm.

differences in color, e.g. *Gastrodia javanica* (Blume) Lindl. f. *thalassina* Yokota, *Eulophia zollingeri* (Rchb. f.) J. J. Sm. f. *viridis* Yokota, *Lecanorchis kiusiana* Tuyama f. *lutea* Y. Sawa, Fukunaga & S. Sawa and *Yoania japonica* Maxim. f. *lutea* Suetsugu & Yagame (Yokota 1998, 1999, Fukunaga *et al*. 2008b, Suetsugu & Yagame 2014). Recently, a greenish form of *G. confusa* Honda & Tuyama f. *viridis* Suetsugu and a whitish form of *G. fontinalis* T. P. Lin f. *albiflora*

Suetsugu were discovered (Suetsugu 2012, 2016).

We found plants resembling *Gastrodia pubilabiata* with a tinge of red color on the whole plant on the floor of a *Phyllostachys edulis* (Carrière) Houz colony (Fig. 1B, C). We investigated the detailed morphology and the habitat of the plants.

Materials and Methods

Both normal *Gastrodia pubilabiata* and the reddish-colored individuals were collected from locality A (Kamo, Tosayamada-cho, Kami city, Kochi). At locality B (Machida, Tosayamada-cho, Kami city, Kochi), only reddish individuals were collected. Five individuals were collected per site per color variation. Sampling was performed twice during the flowering and fruiting period in 2012, 2013 and 2014. The plants were photographed using an Eoss Kiss X5 camera (Canon, Japan). We examined the structure of the lip, column, and perianth. The samples were fixed in 60% ethanol and analyzed using a stereoscopic microscopes (SL-60T, Vixen, Japan).

Carotenoids (0.36 g) were extracted with 80% acetone from one fresh flower (excluding the pedicel) of *Gastrodia pubilabiata* and 0.15 g from the reddish individuals. After centrifuging the extracts for 5 min at 3000 g, absorbance of the supernatants was measured at 470, 646.8 and 663.2 nm using a spectrophotometer (V-550, Jasco, Japan). The concentration of total carotenoids (mg gFW^{-1}) was calculated according to Wellburn (1994). Anthocyanins were extracted with 1% HCl−methanol at room temperature from one fresh flower (excluding the pedicel) of *G. pubilabiata* (0.22 g) and from the reddish individuals (0.08 g). After removal of the insoluble materials with filter paper (No. 2, Toyo Roshi Kaisya, Ltd., Japan), the crude extract was filtrated with DISMIC-13HP (0.45 μm, Toyo Roshi Kaisya, Ltd., Japan). The extracts were analyzed using the LCSS-900 HPLC system (JASCO, Japan) equipped with a multi-wavelength detector MD-910 (JASCO, Japan), and performed with a column of LiChrospher 100 RP-18 (4 × 250 mm, Merck, Germany) and in a column oven CO-965 (35°C, JASCO, Japan) at a flow rate of 1.0 ml/min, detection wavelength of 520 nm. The mobile phase was as follows: a linear gradient elution for 20 min from 25% to 70% solvent B (1.5% H_3PO_4−20% AcOH−25% CH_3CN−H_2O) in solvent A (1.5% H_3PO_4−H_2O). The total phenolic content was determined by the Follin-Ciocalteu method (Singleton *et al*. 1999). The 80% methanol extract (50 μl) was mixed with 50 μl of distilled water and 100 μl of Follin-Ciocalteu reagent (Sigma, USA). After 1 min, 1 ml of 5% Na_2CO_3 was added, and the samples were incubated for 30 min at room temperature. The absorbance was then measured at 760 nm using a spectrophotometer (V-550, Jasco, Japan). Chlorogenic acid was used for the calibration curve. The results are expressed as chlorogenic acid equivalents (CAE), mg CAE gFW^{-1} of the flower extracts.

The Munsell color order system is based on a three-dimensional model depicted in the Munsell color tree. Each color has three qualities or attributes, Hue (color), Value (lightness or darkness), and Chroma (saturation or brilliance). The colors of the floral organs and fruit of five and nine samples of the typical form and the red form, respectively, were evaluated using the Munsell color order system.

Results and discussion

We found three to six reddish plants per year from 2012 to 2014 growing in conjunction with *Gastrodia pubilabiata* at locality A (Fig. 1F). The reddish plants also occurred at locality B, where *G. confusa* was also observed. We found approximately 10 reddish plants per year from 2012 to 2014 at locality B. Both habitats were on the floor of *Phyllostachys edulis* (Carrière) Houz colonies where fallen leaves had accumulated.

The size and morphology of the reddish plants resembled *Gastrodia pubilabiata* except for the number flowers and color of the plants. *Gastrodia pubilabiata* produced 1–6 flowers per stem, while the reddish plants produced 1–12 flowers (mostly >5 flowers) per stem. The reddish plants tended

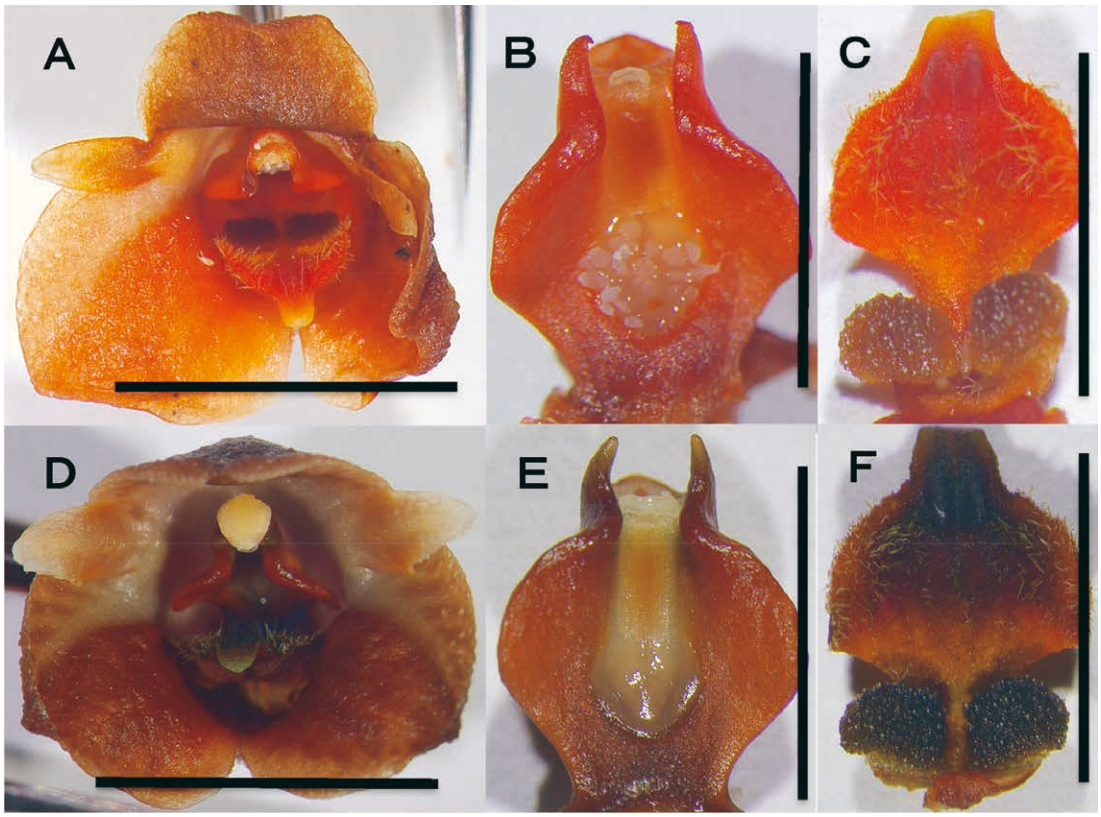

FIG. 2. Floral phenotypes of *Gastrodia pubilabiata* f. *castanea* (A–C) and *G. pubilabiata* (D–F). A, D: Front view of flowering plant. B, E: Column. C, F: lip. Scale bars = 1 cm (A, D), 5 mm (B–C, E–F).

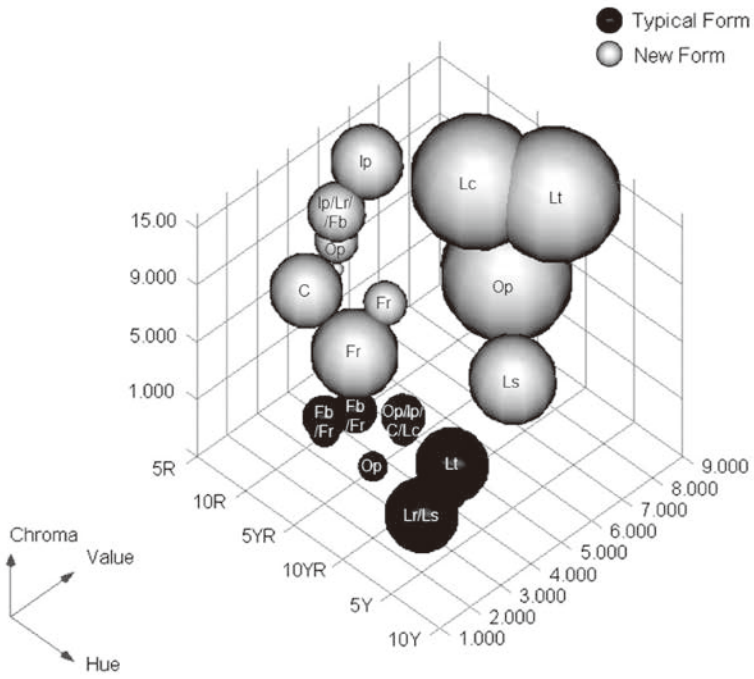

FIG. 3. Dispersion 3d-plot diagram of colors of floral organ/tissue of *Gastrodia pubilabiata* f. *pubilabiata* and f. *castanea*. Color data in Table 1 was used for analysis. Size of the circle reflects sample number. Op, Outer perianth: Ip, Inner perianth: C, Column: Lt, Lip (tip): Lr, Lip (ridged): Ls, Lip (struma): Lc, Lip (center): Fb, Floral bud: Fr, Fruit.

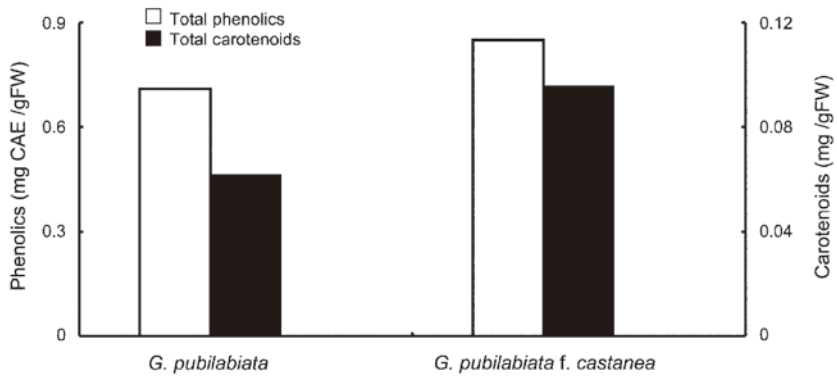

FIG. 4. Total phenolics and total carotenoids in flowers from *Gastrodia pubilabiata* f. *pubilabiata* and f. *castanea*.

FIG. 5. A: *Drosophila* (*Sophophora*) sp. visiting flower of *Gastrodia pubilabiata* f. *castanea*. B: *Drosophila* (*Sophophora*) sp. with pollinium on its back. Scale bars = 1 cm.

produce more flowers than *G. pubilabiata*. *Gastrodia pubilabiata* has a blackish brown perianth, blackish brown calli on the ventral surface of the lip, purplish brown lip apex, and blackish brown fruits (Fig. 1A, D, F, G, Fig. 2D, E, F). In contrast, the reddish plants had a reddish brown perianth, greenish brown calli, reddish yellow lip apex, and reddish brown fruit (Fig. 1B, C, D, E, G, Fig. 2A, B, C). In fruit, the reddish individuals produced redder fruits and distal part of the pedicel in comparison with *G. pubilabiata* (Fig. 1G). To evaluate the color difference between the reddish plants and *G. pubilabiata*, we used a Munsell color chart (Munsell 1977). The observations revealed that the colors of the floral organs and fruit clearly differed and did not overlap between the two types of plants (Table 1, Fig. 3).

To determine the cause of the color difference, we examined the major pigments; carotenoids, anthocyanins, and phenolics. Carotenoids are major pigments providing yellow or red coloration. Orange or red coloration is produced by a combination of anthocyanins and carotenoids or by an increased accumulation of carotenoids

TABLE 1. Color difference between two forms evaluated by Munsellcolor order system. Sample number is shown in parentheses.

Tissue/organ	Typical form	New form
Outer perianth	10YR3/4(3), 10YR2/2(2)	10R4/8(1), 10R3/8(5), 10R4/10(3)
Inner perianth	10YR3/3(2), 10YR3/4(3)	10R5/14(5), 10R4/12(4)
Column	10YR3/4(5)	10R3/8(6), 10R3/8(3)
Lip (tip)	5Y3/3(5)	10YR8/12(9)
Lip (ridged)	5Y2/1(5)	10R4/12(9)
Lip (struma)	5Y2/1(3), 5Y3/1(2)	5Y5/6(6), 5Y5/6(3)
Lip (center)	10YR3/4(5)	5YR7/12(9)
Floral bud	5YR2/3(3), 5YR3/4(2)	10R4/12(7), 10R4/10(2)
Fruit	5YR3/2(3), 5YR2/2(2)	5YR3/6(6), 5YR4/8(3)

(Kishimoto *et al.* 2007). Phenolics, other than anthocyanins, can affect coloration in combination with anthocyanins (Cheynier *et al*., 2013). The flowers of the reddish individuals had a higher concentration of total carotenoids and total phenolics than the flowers of *Gastrodia pubilabiata* (Fig. 4). No anthocyanins were detected in the flowers (data not shown). Because the flowers of *G. pubilabiata* did not have anthocyanins, phenolics other than anthocyanins could not be responsible for the color differences. The change in flower color in the reddish individuals appears to be due to increased carotenoid content.

The reddish plants clearly differed from *Gastrodia pubilabiata* in color, but not in morphology. Therefore, we describe the reddish plants as a new form of *G. pubilabiata*. The morphology and the color of the red form have been stable for at least 6 years (personal communication with Mr. Takeuchi). The reddish plants grow alongside the typical *G. pubilabiata*. At their closest, the different forms flowered at a distance of approximately 30 cm from each other at locality B, indicating that they grow in similar environments and that the color of the reddish plants is unlikely the result of different environmental conditions.

The type locality is in close proximity to the large river, Monobe-gawa, and as such, the environmental conditions of the type locality may be altered due to flooding. Furthermore, wastes from Shiitake mushroom growing facilities are clearly present around the type locality. Careful monitoring should be practiced to maintain the environment.

Because the flowering time of *Gastrodia pubilabiata* is brief and the flowers are produced near the soil surface, it was difficult to find flowering plants essential for identification. A detailed study of the distribution for *G. pubilabiata* is therefore insufficient. Many recent studies have reported new habitats for *G. pubilabiata*, not only in Shikoku, including Kochi Prefecture (Fukunaga *et al*. 2008a), but also throughout Japan (Environment Agency of Japan 2000). It is difficult to distinguish the two forms by color in dried specimens or specimens preserved in alcohol. Colored forms may be misidentified as the typical form. It is possible that the red form is widely distributed in other regions. To understand the distribution of the red form, careful observations of living plants are necessary.

Interestingly, we confirmed that many individuals of *Drosophila* (*Sophophora*) spp. visit the flowers of the red form (Fig. 5A). Some flies were observed to have pollinia on their back (Fig. 5B). Species of *Drosophila* have been reported to be responsible pollination of some species of *Gastrodia* (Suetsugu & Kato 2014, Martos *et al*. 2015) that attract flies by mimicking the scent of fermented mushrooms (Suetsugu & Kato 2014, Suetsugu 2015b). It is well known that floral color is responsible for attracting pollinators (Weiss 1991). The reddish color of *G. pubilabiata* may also work as an effective pollinator attractant. It is worth testing whether the frequency of pollinator visits and the assemblage of pollinators on the red form differ from the typical species.

Taxonomic treatment

Gastrodia pubilabiata Y. Sawa f. **castanea** Fukunaga & S. Sawa, **forma nov.** —Fig. 1B–C, E, Fig. 2A–C.

Compared to the typical form, forma *castanea* produces reddish brown flowers, stems and fruit, greenish brown calli at the base of the lip and a reddish yellow lip apex. The typical form produces blackish brown calli at the base of the lip and a blackish brown lip apex.

Japanese name. Bengara-yatsushiroran. (Etymology: The name comes from the Japanese traditional color Bengara).

Typus. JAPAN, Shikoku, Kochi Pref. Kami-shi, Tosayamada-cho, Machida, along Monobe River, 21 Sep. 2014, *H. Fukunaga s.n*., (Holotype in MBK), 8 Oct. 2012, *H. Fukunaga s.n*. (paratype in MBK).

Additional specimen. 8 Oct. 2012 *H. Fukunaga s. n*. (MBK)

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